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U. S. DEPARTMENT OF AGRICULTURE,

BUREAU OF ANIMAL INDUSTRY.—BULLETIN 154.

A. D. MELVIN, CHIEF OF BUREAU.

METHODS OF CLASSIFYING THE LACTIC-ACID BACTERIA.

UNIVERSITY OF CALIFORNIA AT LOS ANGELES
MAR 16 1938
BY

BY

LORE A. ROGERS,
Bacteriologist, Dairy Division,

AND

BROOKE J. DAVIS,
Assistant, Dairy Division.

WASHINGTON:
GOVERNMENT PRINTING OFFICE.
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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Animal Industry,
Washington, D. C., March 22, 1912.

Sir: I have the honor to transmit for publication as a bulletin of this bureau the accompanying manuscript entitled "Methods of Classifying the Lactic-Acid Bacteria," by Messrs. Lore A. Rogers and Brooke J. Davis, of the Dairy Division of this bureau.

There has hitherto been felt a need by dairy bacteriologists and others of a classification of the lactic-acid bacteria into naturally related groups by means of characters that can be determined with reasonable accuracy and in a manner ordinarily available. This paper describes the study of about 150 cultures isolated from milk, butter, and cheese, derived from various parts of the country, with the object of laying the basis for a satisfactory classification.

Respectfully,

A. D. Melvin,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

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METHODS OF CLASSIFYING THE LACTIC-ACID BACTERIA.

INTRODUCTION.

The grouping of bacteria according to their action on any one specific substance usually brings together bacteria related in that one characteristic only but entirely unrelated in other respects. It is, however, sometimes convenient from a technical standpoint to group bacteria in this way. The bacteria concerned in the souring of milk have been so grouped for so long that many people have come to consider them as a division by themselves and their relation to other bacteria has been little considered. The bacteria taking part in the souring of milk may be readily divided into four general groups.

Group I includes those bacteria which sour milk without peptonization or gas formation; they grow poorly on artificial media and fail to liquefy gelatin. Morphologically they show some variation, usually appearing as a coccus or very short bacillus in pairs or in chains of varying lengths. The bacteria of this group are the ones ordinarily designated as the lactic-acid bacteria and have been described under various names. They have a very general distribution and their presence in milk is so constant that they may be considered as normal inhabitants of this medium.

Group II includes the bacteria forming an acid curd with evolution of gas. This embraces varieties of *Bacillus coli* and *Bacterium aerogenes* or the *Bacillus acidi lactici* of Heuppe. The members of this group are readily distinguished from those of Group I by their abundant growth on artificial media, the vigorous evolution of gas, and the marked difference in their morphology. An examination of milk usually reveals their presence in small numbers, but their number is increased by the influence of high temperatures or insanitary conditions under which the milk has been collected or held.

Group III includes those bacteria forming an acid curd which is subsequently partially peptonized. The bacteria of this group have been little studied in their relation to milk. It will be shown that this description applies to varieties only distantly related to our Group I as well as to some closely connected with this type.

Group IV includes the high-acid-forming bacteria of which the *Bacillus bulgaricus* is the type. This organism is distinguishable from

those of the preceding groups by its slender rodlike form, its characteristic colonies on agar, its inability to grow in ordinary artificial media, and its growth in the presence of free acid. *Bacillus bulgaricus* has been studied in its relation to the fermented milks extensively used in Turkey and the neighboring countries. It has recently been shown[a] [1, 2, 3], that it is very widely distributed and may be isolated from almost any sample of mixed milk. Its growth at normal temperatures is so slow that it is improbable that it is a factor in the ordinary souring of milk.

It is obvious that these groups are connected only by their ability to ferment lactose to acid and the consequent precipitation of the casein. Groups I, II, and IV are evidently related to each other in no other way. The identity of the bacteria of Group III in so far as they occur as milk bacteria has not been established. The need of work on methods of classification rather than on descriptions of new varieties or rearrangement of old names and descriptions is exemplified by the confused nomenclature of the bacteria included under Group I. We find in the literature such names as *Bacterium lactis acidi*, *Bacillus lactis acidi*, *B. acidi lactici*, *B. guntheri*, and *Streptococcus lacticus*, all of which, so far as can be determined by published descriptions, may be included in Group I. These names are based on variations in morphology, differences in growth on artificial media, on the rate of acid formation in milk, and various other characteristics of doubtful significance and uncertain stability. In the light of recent investigations these names and their accompanying descriptions have little more than a historic interest. They represent the attempt to establish types by the study of an isolated individual organism with little regard to its relation to other similar individual organisms.

Approached from the standpoint of the dairy bacteriologists, the lactic-acid bacteria have been considered as a sharply defined group peculiar to milk. The students of pathogenic bacteria have been inclined to look on them as a variety of some of the pus-forming streptococci.

The opinion that the term lactic-acid bacteria covers a group of species or varieties and that the names and descriptions already published do not represent the true grouping is reflected in the frequent attempts to establish means of separating the group into stable species or varieties. McDonnell[4] attempted to do this, basing his descriptions largely on the effect on milk. A somewhat similar course was followed by Weigmann.[5] Müller[6] found a correlation in the solution of red blood corpuscles and the agglutination of immune sera by the streptococci of sour milk and certain pathogenic streptococci,

[a] The reference figures relate to the list of references to literature at the end of this bulletin.

and considered that this indicated a relationship. Lohnis[7] has made a classification of the lactic-acid bacteria based largely on gas formation, coagulation of milk, formation of slime, and liquefaction of gelatin. Conn, Esten, and Stocking[8] have used in their descriptions the action on milk and the usual culture characteristics. Heinemann[9] as a result of his studies on the bacteria of sour milk, excludes the name of *Bacillus acidi lactici*, and concludes that aside from the part taken by *B. aerogenes* and possibly by *B. coli* the spontaneous souring of milk is brought about by the *Streptococcus lacticus* of Kruse, an organism identical with the common streptococci of sewage and pathological conditions. He bases this conclusion on the similarity in morphological, cultural, and pathogenic properties.

All of this work and much more of a similar nature adds little to the systematic arrangement of the lactic-acid bacteria. It is generally admitted that it is difficult to identify cultures of any but the best known and most carefully studied bacteria by the published descriptions. Cultures which seem identical when written descriptions are compared are found to be distinct when they are grown side by side under uniform conditions. This error in identification is due partly to the difficulty of conveying the appearance of an object by words, but in a larger degree to the instability and unessential nature of some of the characters employed in separating one bacterial species or variety from another. The inadequacy of the ordinary methods is particularly felt when one attempts a description of the lactic-acid bacteria. The cells are small and the morphological differences are uncertain and inconstant. The growth on solid media is scanty and devoid of distinguishing characteristics. While many of the physiological tests which are found of great value in some groups fail when applied to the lactic-acid bacteria, others, notably the acid fermentations of sugars, offer a possible means of differentiation for members of this group.

Variations in the ability to ferment sugars have been observed, but the use of these variations in classifying or identifying cultures has been limited for two reasons. There has been a belief that the fermentative power was not constant; that this property could be lost or acquired so readily that it could not be used to differentiate one culture from another with any certainty. The more common objection, however, is that the separation on the basis of sugar fermentation divides the lactic-acid bacteria and others possessing the same general characteristics, not into natural groups, but into innumerable varieties.

The use of tests of this kind in the usual way by which the fermentation of, or failure to ferment, a certain substance sets the culture so reacting apart from all other cultures gives an endless dichotomy

limited only by the number of test substances. Consequently, the ordinary use of sugars has increased rather than diminished the confusion now existing in the classification of the zymogenic bacteria. It is obvious that what is required in systematic bacteriology is not descriptions of new species or a rearrangement of names, but the establishment of means of classification applicable technically and correct biologically. No one basis of classification can be used for all groups of bacteria, but certain fundamental principles should govern any method of arrangement. Two of the most obvious principles on which the selection of characters for classification should be based may be stated as follows: The characters should be constant; they should be so selected that they show real biological relationships. In other words, bacteria should be arranged by means of characters that can be determined with reasonable accuracy and by means ordinarily available into groups whose members are related naturally rather than by artificial bonds, and these characters should be so constant and so distinctive that identical organisms can always be placed in the same group.

This paper records an attempt to determine which of the characters exhibited by the lactic-acid bacteria fulfill these conditions. No attempt has been made to classify or name any members of this group or to fix its place in the general bacteriological system.

THE SIGNIFICANT CHARACTERS OF THE LACTIC-ACID BACTERIA.

The morphology, staining reactions, cell grouping, cultural characters, and growth in milk were considered, but more attention was given to the fermentation tests. We studied about 150 cultures isolated from milk, butter, and cheese obtained from various parts of the country. This collection included, in addition to the typical milk-curdling, nonliquefying, lactic-acid bacteria, a number of cultures curdling milk with subsequent digestion and which formed on gelatin plates small saucer-shaped liquefactions surrounding a solid colony.

We have determined on these cultures the morphology, Gram's stain, cell grouping, in many cases formation of capsule, the nature and amount of growth on lactose-agar slopes and in gelatin stabs, the rate of liquefaction of gelatin, the nature of growth in broth, growth in milk, the reduction of nitrates and of neutral red, and the formation of acid in broth containing various test substances. In these fermentation tests we have used the sugars lactose, dextrose, galactose, saccharose, and raffinose, the alcohols mannite and glycerin, and the polysaccharid inulin. The results of these determinations are given in Table 1.

TABLE 1.—*Significant characteristics of acid-forming bacteria derived from milk, butter, and cheese.*

Culture.	Agar.		Capsule.	Gram stain.	Cloudiness in broth.	Reduction of neutral red.	Curdling of milk.	Reduction of nitrates.	Mm. gelatin liquefaction 30 days at 20°.	Per cent lactic acid in broth after 7 days at 30° C.							
	Good.	Scanty.								Dextrose.	Lactose.	Saccharose.	Glycerin.	Mannite.	Galactose.	Raffinose.	Inulin.
6es	....	+	+	+	+	....	+	−	0	0.360	0.319	0.000	0.000	0.000	0.198	0.000	0.000
6ex	....	+	+	+	+	....	+	−		.612	.360	.540	.000	.000	.234	.009	.009
6ez	....	+	....	+	+	....	+	−	0	.351	.450	.522	.000	.009	.216	.000	.018
6fa	....	+	....	+	+	....	+	−	0	.382	.423	.000	.000	.000	.324	.000	.009
6fb	....	+	+	+	+	....	+	−	0	.405	.378	.477	.000	.000	.378	.000	.000
6fl	....	+	....	....	+	....	....	+	0	.288	.315	.405	.200	.279	.081	.171	.000
6fn	....	+	+	+	+	....	+	−	0	.364	.316	.000	.000	.000	.288	.000	.009
6fr	....	+	....	+	+	....	+	−	0	.668	.315	.000	.000	.000	.270	.000	.009
7b	....	+	....	+	+	−	+	−	0	.558	.378	.531	.000	.000	.297	.000	.009
7c	....	+	+	....	+	−	+	−	0	.400	.432	.000	.000	.000	.342	.000	.000
7d	....	+	....	....	+	−	+	−	0	.378	.414	.450	.027	.000	.283	.000	.009
7f	....	+	....	+	−	....	+	−	0	.693	.378	.405	.000	.000	.306	.000	.009
7g	....	+	....	+	+	....	+	−	0	.365	.414	.000	.000	.000	.315	.000	.000
7i	....	+	....	+	−	....	+	−	0	.324	.414	.000	.018	.000	.342	.000	.009
7j	....	+	....	+	−	....	+	−	0	.274	.387	.405	.000	.000	.054	.000	.009
7k	....	+	....	+	+	−	+	−	0	.369	.396	.000	.000	.000	.342	.000	.009
7n	....	+	....	+	+	....	+	−	0	.493	.333	.000	.000	.000	.405	.000	.018
7o	....	+	....	+	+	....	+	−	0	.423	.387	.000	.000	.000	.423	.009	.000
7p	....	+	....	+	+	....	+	−	0	.378	.378	.000	.000	.018	.397	.000	.009
7q	....	+	....	+	+	....	+	−	0	.360	.360	.000	.000	.252	.351	.000	.009
7s	....	+	....	+	+	....	+	−	0	.360	.369	.531	.000	.000	.189	.000	.000
7t	....	+	....	+	+	....	+	−	0	.585	.405	.526	.000	.252	.396	.009	.018
7u	....	+	....	+	+	....	+	−	0	.383	.324	.594	.000	.225	.242	.000	.018
7w	....	+	....	+	+	−	+	−	0	.306	.324	.009	.000	.000	.324	.000	.000
7x	....	+	....	+	+	....	+	−	0	.594	.378	.000	.000	.324	.369	.000	.009
7y	....	+	....	+	+	−	+	−	0	.504	.369	.000	.000	.000	.315	.000	.000
7z	....	+	....	....	−	−	+	−	0	.288	.171	.000	.000	.360	.108	.000	.009
7aa	....	....	....	+	+	....	−	+	0	.540	.306	.000	.283	.283	.225	.000	.000
7ab	+	....	....	....	−	−	+	−	0	.000	.162	.009	.018	.000	.378	.009	.000
7ad	....	+	....	+	+	−	+	−	0	.522	.351	.000	.000	.000	.216	.000	.009
7ae	....	+	....	+	−	....	+	−	0	.396	.360	.000	.000	.000	.306	.000	.009
7af	....	+	....	+	−	....	+	−	0	.351	.369	.000	.000	.000	.099	.....	.000
7ag	....	+	+	+	+	....	+	−	0	.567	.351	.000	.000	.000	.252	.000	.009
7ah	....	+	....	+	+	....	−	−	0	.432	.378	.252	.000	.207	.000	.009	.009
7ai	....	+	....	+	....	....	+	−	0	.603	.360	.000	.009	.000	.414	.000	.009
7aj	....	+	....	....	....	....	−	−	0	.594	.270	.000	.027	.324	.153	.000	.009
7ak	+	....	....	+	+	....	+	+	0	.468	.306	.000	.234	.315	.297	.000	.009
7ao	+	....	....	−	....	....	+	−	....	.585	.495	.423	.072	.018	.234	.000	.000
7ap	....	+	....	+	....	....	+	−	....	.630	.369	.027	.000	.081	.234	.000	.009
7aq	....	....	....	....	....	....	....	....	....	.360	.261	.....	.000	.....	.....	.027	.009
7ar	....	+	....	+	....	....	+	−	....	.....	.279	.378	.000	.342	.216	.009	.009
7as	....	+	....	+	+	....	+	−	....	.702	.360	.000	.072	.009	.234	.000	.036
7ay	....	+	+	+	+	....	+	−	....	.513	.423	.000	.009	.009	.189	.000	.000
7ba	....	+	....	+	+	....	+	−	....	.702	.369	.054	.000	.018	.243	.000	.000
7be	....	+	....	....	−	−	+	−	0	.288	.171	.009	.342	.000	.171	.000	.018
7bg	....	+	....	....	−	−	+	−	0	.180	.279	.387	.036	.045	.252	.009	.009
7bi	....	....	....	....	+	−	+	−	0	.396	.342	.000	.009	.000	.360	.000	.000
7bj	....	....	....	....	+	−	+	−	0	.396	.324	.000	.009	.063	.396	.018	.000
7bn	....	....	....	....	+	−	+	−	0	.306	.189	.000	.009	.000	.288	.018	.000
7bp	....	....	....	....	−	−	+	−	0	.294	.207	.000	.009	.063	.306	.000	.000
7bq	....	....	....	....	....	−	+	−	....	.306	.036	.000	.000	.....	.234	.018	.000
7br	....	....	....	....	−	−	+	−	0	.270	.342	.000	.009	.000	.378	.018	.000
7bs	....	....	....	....	+	−	+	−	0	.332	.396	.000	.000	.315	.396	.018	.009
7bt	....	....	....	....	−	−	+	−	0	.306	.297	.000	.000	.000	.360	.000	.018
7bv	....	....	....	....	....	−	+	−	0	.423	.360	.000	.027	.018	.333	.000	.216
7bx	....	....	....	....	....	−	+	−	0	.360	.216	.018	.054	.009	.288	.000	.009
7by	....	....	....	....	+	−	....	−	0	.549	.193	.009	.000	.018	.441	.009	.000
7bz	....	....	....	....	....	−	+	−	0	.297	.387	.693	.036	.000	.000	.000	.000
7ca	....	....	....	....	+	−	+	−	0	.441	.256	.000	.000	.036	.306	.000	.000
7cb	....	....	....	....	+	−	+	−	0	.378	.144	.027	.000	.018	.324	.000	.027
7cc	....	....	....	....	+	+	+	+	11	.360	.221	.288	.009	.397	.351	.009	.387
7cd	....	....	....	....	−	−	+	−	0	.558	.297	.009	.000	.018	.414	.000	.000
7ce	....	....	....	....	−	−	+	−	....	.351	.144	.225	.018	.009	.261	.000	.000
7cf	....	....	....	....	+	−	+	....	....	.225	.103	.009	.009	.018	.324	.000	.....
7cg	....	....	....	....	−	+	+	−	0	.495	.297	.288	.153	.486	.333	.000	.000
7ch	....	....	....	....	+	−	+	−	0	.603	.248	.000	.000	.018	.414	.045	.009
7ci	....	....	....	....	−	+	+	−	0	.432	.260	.324	.036	.378	.423	.000	.000
7cj	....	....	....	....	−	−	+	−	0	.621	.270	.018	.018	.000	.243	.009	.000
7ck	....	....	....	....	....	−	+	−	0	.261	.297	.099	.036	.009	.302	.000	.018
7cl	....	....	....	....	....	+	+	−	0	.540	.648	.036	.045	.018	.315	.000	.000
7cm	....	....	....	....	+	−	+	−	0	.513	.261	.063	.000	.009	.306	.000	.018

TABLE 1.—*Significant characteristics of acid-forming bacteria derived from milk, butter, and cheese*—Continued.

Culture.	Agar.		Capsule.	Gram stain.	Cloudiness in broth.	Reduction of neutral red.	Curdling of milk.	Reduction of nitrates.	Mm. gelatin liquefaction 30 days at 20°.	Per cent lactic acid in broth after 7 days at 30° C.							
	Good.	Scanty.								Dextrose.	Lactose.	Saccharose.	Glycerin.	Mannite.	Galactose.	Raffinose.	Inulin.
7cn	....	....	....	....	+	−	+	−	0	0.378	0.234	0.000	0.000	0.018	0.477	0.000	0.000
7co	....	....	....	....	+	−	+	−	0	.351	.206	.099	.000	.027	.342	.009	.018
7cp	....	....	....	....	+	−	+	−	0	.576	.274	.027	.000	.000	.441	.225	.000
7cq	....	....	....	....	+	−	+	−	0	.576	.225	.009	.000	.009	.450	.000	.000
7cr	....	....	....	....	+	+	+	−	0	.459	.265	.009	.009	.243	.522	.000	.009
7cs	....	....	....	....	+	−	+	−	0	.522	.243	.000	.009	.117	.405	.027	.036
7ct	....	....	....	....	+	−	+	−	0	.684	.297	.000	.009	.036	.405	.000	.009
7cu	....	....	....	....	....	+	+	−	0	.261	.108	.009	.009	.009	.108	.000	.018
7cv	....	....	....	....	....	+	+	−	0	.351	.414	.531	.162	.531	.279	.630	.270
7cx	....	....	....	....	....	+	+	−	0	.316	.441	.459	.198	.549	.216	.630	.000
7cy	....	....	....	....	....	+	+	−	0	.423	.018	.029	.225	.504	.081	.648	.000
7cz	....	....	....	....	....	....	+	−	0	.495	.372	.009	.045	.045	.279	.018	.018
7da	....	....	....	....	....	+	+	−	0	.387	.423	.549	.117	.531	.234	.603	.306
7dc	....	....	....	....	....	−	+	−	0	.405	.432	.018	.099	.018	.036	.054	.000
7dd	....	....	....	....	....	+	+	−	0	.316	.396	.459	.045	.009	.378	.000	.000
7de	....	....	....	....	....	+	+	−	0	.279	.....	.450	.198	.567	.252	.630	.252
7df	....	....	....	....	....	+	+	....	0	.369	.423	.477	.252	.558	.279	.648	.216
7dg	....	....	....	....	....	+	+	−	0	.333	.414	.522	.234	.558	.324	.630	.288
7dh	....	....	....	....	....	+	+	−	0	.369	.441	.531	.261	.558	.252	.603	.279
7dl	....	....	....	....	....	+	+	−	0	.423	.414	.549	.162	.549	.279	.567	.288
7dj	....	....	....	....	....	+	+	−	0	.225	.405	.468	.234	.558	.243	.487	.261
7dk	....	....	....	....	....	+	+	−	0	.171	.405	.504	.198	.549	.261	.639	.000
7dl	....	....	....	....	....	+	+	−	0	.396	.450	.540	.234	.595	.279	.648	.306
7dm	....	....	....	....	....	+	+	−	0	.405	.009	.018	.000	.054	.153	.703	.000
7dn	....	....	....	....	....	+	+	−	0	.396	.432	.558	.027	.531	.288	.414	.297
7do	....	....	....	....	....	+	+	−	0	.288	.414	.522	.315	.549	.198	.676	.342
7dp	....	....	....	....	....	+	+	−	0	.216	.414	.540	.225	.567	.288	.639	.108
7ds	....	....	....	....	....	+	+	−	....	.432	.423	.549	.198	.558	.558	.261	.630
7du	....	....	....	....	....	+	+	−	0	.351	.441	.468	.180	.540	.234	.676	.306
7dv	....	....	....	....	....	+	+	−	0	.288	.441	.369	.216	.549	.270	.756	.270
7dw	....	....	....	....	....	+	+	+	0	.414	.414	.612	.252	.594	.288	.657	.306
7dx	....	....	....	....	....	+	+	−	0	.334	.423	.423	.180	.540	.198	.630	.000
7dy	....	....	....	....	....	+	+	−	0	.378	.441	.414	.189	.558	.270	.648	.000
7dz	....	....	....	....	....	−	+	−	0	.594	.432	.000	.000	.000	.288	.000	.000
10av	....	+	....	+	+	....	+	−	0	.531	.369	.000	.000	.000	.351	.000	.027
10aw	....	+	+	+	−	....	+	−	0	.378	.351	.000	.000	.054	.216	.000	.000
10ax	....	+	....	+	−	....	+	−	0	.522	.405	.000	.000	.018	.351	.000	.000
10bb	....	+	+	+	+	....	+	−	0	.477	.351	.540	.000	.000	.270	.000	.000
10bd	....	+	+	+	+	....	+	−	0	.486	.369	.000	.000	.009	.171	.000	.009
10be	....	+	+	+	+	....	+	−	0	.....	.387	.000	.018	.000	.315	.000	.009
10bg	....	+	+	+	+	....	+	−	0	.495	.405	.000	.018	.000	.342	.000	.000
10bh	....	+	+	+	+	....	+	−	0	.468	.387	.000	.000	.000	.360	.009	.000
10bi	....	+	+	+	+	....	+	−	0	.432	.333	.000	.000	.000	.143	.000	.000
10bo	....	+	+	+	+	....	+	−	0	.603	.343	.000	.018	.000	.369	.000	.000
10br	....	+	+	+	+	....	....	−	0	.495	.369	.072	.009	.000	.225	.009	.009
10bt	....	+	....	+	−	−	+	−	0	.720	.045	.000	.000	.000	.036	.000	.009
10bl	....	+	+	+	−	−	....	−	0	.324	.108	.585	.000	.369	.063	.000	.009
13c	+	....	+	+	+	+	+	−	9	.234	.171	.027	.048	.072	.099	.000	.009
13f	....	+	+	+	+	+	+	−	14	.369	.216	.274	.207	.191	.239	.009	.018
13h	....	+	+	+	+	+	+	−	26	.324	.194	.009	.216	.406	.234	.000	.018
13i	+	....	+	+	+	−	+	−	5	.220	.144	.014	.027	.004	.099	.000	.009
13m	....	+	+	+	+	+	+	−	24	.360	.270	.000	.162	.243	.175	.000	.000
13o	+	....	+	+	+	−	+	−	21	.230	.144	.252	.072	.004	.036	.000	.018
13r	+	....	+	+	+	−	+	+	6	.211	.126	.153	.054	.000	.072	.000	.018
13t	....	+	....	+	+	−	+	−	10	.165	.144	.285	.081	.297	.144	.000	.036
13u	+	....	+	+	....	+	+	+	18	.270	.077	.351	.180	.135	.146	.027	.036
13w	....	+	....	+	....	+	+	−	13	.203	.275	.351	.086	.162	.198	.018	.018
13x	....	+	....	+	....	....	+	−	15	.149	.176	.198	.207	.230	.203	.018	.009
13y	+	....	+	....	+	−	+	+	25	.432	.316	.369	.315	.216	.135	.000	.009
13z	+	....	+	+	+	−	−	+	15	.099	.234	.297	.234	.297	.144	.000	.009
13aa	+	....	....	+	+	+	+	−	8	.207	.171	.036	.054	.018	.058	.009	.009
13ab	+	....	+	+	+	−	+	−	7	.243	.175	.192	.027	.045	.063	.045	.018
13ac	+	....	....	+	+	−	....	+	9	.250	.153	.045	.045	.036	.081	.000	.000
13ad	....	+	....	+	+	−	+	−	0	.495	.324	.018	.036	.018	.256	.027	.000
13ag	....	+	....	+	+	−	+	−	6	.652	.355	.018	.045	.009	.261	.000	.000
13ah	+	....	....	+	+	−	+	−	5	.216	.153	.036	.009	.009	.075	.018	.000
13ai	....	+	....	+	+	−	+	−	0	.633	.324	.018	.045	.018	.279	.000	.018
13aj	+	....	....	....	+	+	+	+	6	.171	.171	.045	.000	.000	.054	.000	.000
13ak	+	....	....	....	....	−	+	+	13	.180	.117	.045	.121	.027	.090	.036	.....
13am	+	....	....	....	....	−	+	−	14	.198	.121	.054	.072	.018	.081	.054	.....
13ap	+	....	....	+	....	....	....	+	5	.198	.144	.090	.036	.027	.287	.018	.....

TABLE 1.—*Significant characteristics of acid-forming bacteria derived from milk, butter, and cheese*—Continued.

Culture.	Agar.		Capsule.	Gram stain.	Cloudiness in broth.	Reduction of neutral red.	Curdling of milk.	Reduction of nitrates.	Mm. gelatin liquefaction 30 days at 20°.	Per cent lactic acid in broth after 7 days at 30° C.						
	Good.	Scanty.								Dextrose.	Lactose.	Saccharose.	Glycerin.	Mannite.	Galactose.	Raffinose.	Inulin.
13ar	....	....	....	....	....	−	+	−	....	.....	0.198	0.027	0.027	0.009	0.153	0.036	.....
13at	+	....	....	....	....	−	+	−	18	0.162	.171	.054	.054	.027	.117	.063	.....
13av	+	....	....	....	....	−	+	−	11	.171	.145	.045	.045	.000	.063	.036	.....
13aw	+	....	....	+	....	−	+	−	7	.117	.135	.090	.036	.009	.090	.036	.....
13ax	+	....	....	....	....	−	+	+	6	.126	.181	.000	.027	.000	.090	.009	.....
13be	+	....	....	+	....	−	+	+	12	.216	.135	.054	.090	.000	.099	.027	.....
13bg	+	....	....	....	....	−	+	+	13	.189	.136	.036	.072	.009	.054	.072	.....
13bk	....	....	....	....	....	+	+	+	....	.297	.207	.126	.009	.009	.189	.018	.....
13bl	....	....	....	....	....	−	+	+	....	.234	.117	.054	.036	.027	.108	.081	.....

MORPHOLOGY.

The cultures examined showed four more or less distinct types. The liquefying group included a number of cultures of micrococci, with frequent grouping in tetrads. This was associated with good growth on agar and certain fermentative reactions which made them easily distinguishable from the liquefying cultures with the morphology of the typical lactic bacteria. The nonliquefiers, excepting a few cultures of micrococci, showed three variations. Cells may be nearly or quite round. When in this condition they are usually found in chains of four or more cells. Single cells or pairs of cells are almost always oval and sometimes are distinctly of the bacillus type. A slight variation is sometimes found in that the cells are somewhat pointed at one end. All of these types can usually be found in the same culture and not infrequently in the same microscopic field.

The question of the classification of the lactic-acid bacteria as cocci or as bacilli has been much debated and is yet in an unsettled condition. The formation of chains, on which much emphasis is placed by some writers, is not pertinent, as the tendency to form chains is as common among the bacilli as among the streptococci. In our present state of knowledge the proper placing of these bacteria is largely a question of opinion or of definition. No satisfactory settlement can be reached until we have sufficient knowledge to establish the natural relations of the lactic-acid bacteria with other groups. Even in this case it is probable that close relationship will be traced on the one hand with groups that are distinct cocci and on the other with groups that are unquestionably bacilli.

It may be stated in this connection that one of our cultures (7dv), not differing morphologically from other cultures of the typical

lactic type, was actively motile. This culture was pronounced by Dr. Heinemann to be a typical *Streptococcus lacticus*, except that it did not curdle milk promptly.

All of these cultures stain readily and are Gram positive. A capsule could usually be demonstrated if the test was repeated under varying conditions, indicating that it is formed only under certain circumstances. The circumstances under which a capsule was found indicated that it was in some way connected with the acidity of the culture.

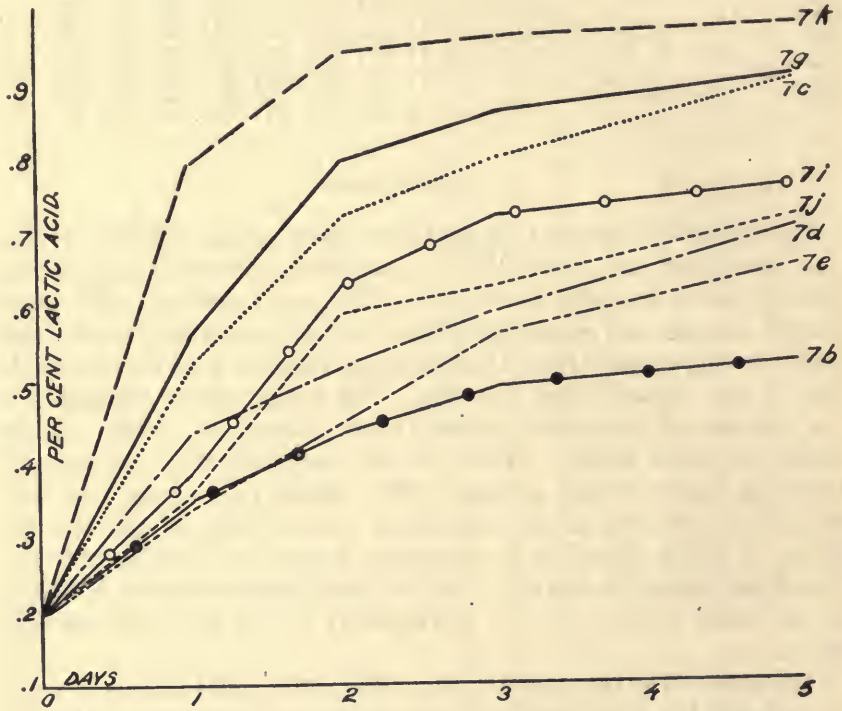

FIG. 1.—Rate of acid formation in milk at 30° C. by cultures freshly isolated from milk.

GROWTH ON SOLID MEDIA.

Little need be said under this head, although the growth of the lactic-acid bacteria on agar and gelatin has been described in great detail. The growth is always scanty, and the variations, which are very slight, are due more to differences in the chemical and physical condition of the medium than to varietal distinction.

Some real variation may be observed in the size of mature gelatin colonies, but this is so influenced by the medium and the number of colonies on the plate that it is of little value. Variations of this kind are probably merely expressions of a tolerance or intolerance to certain conditions which could be determined with much greater

accuracy by other methods. The reaction of a particular lot of gelatin may produce large colonies of one culture while it limits the growth of another culture to colonies of almost microscopic size.

GROWTH IN MILK.

The time required to curdle milk under definite conditions has been employed almost universally in describing lactic-acid bacteria, although it is generally admitted that this property is variable, especially after the culture has been grown under artificial conditions. This variation is illustrated by figures 1 and 2.

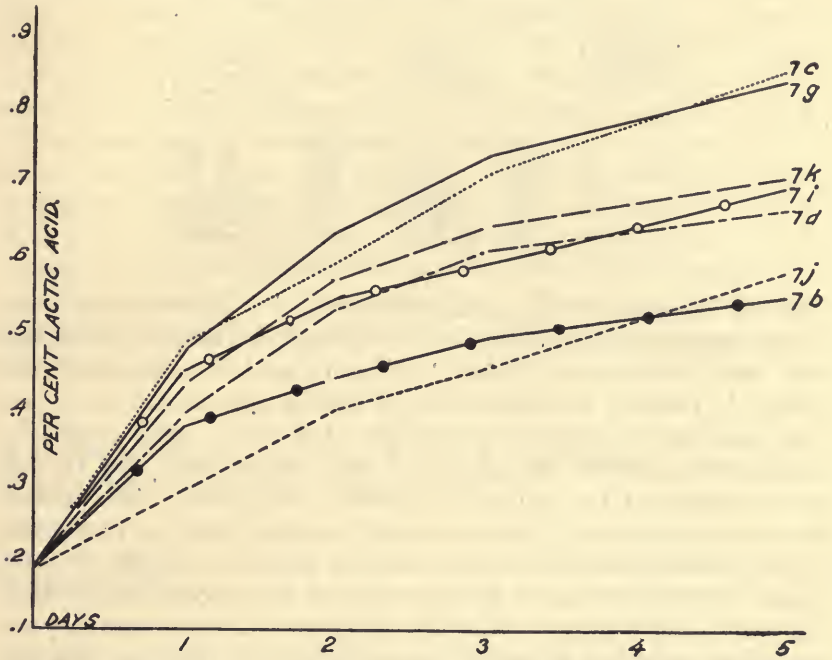

FIG. 2.—Rate of acid formation in milk at 30° C. after 26 generations (2 years) on actose-agar.

Figure 1 shows a variation in the freshly isolated cultures from 7b, which failed to curdle milk in 5 days, to 7k, which curdled milk promptly, forming nearly 1 per cent of acid in 48 hours. Two years' growth under uniform conditions reduced these differences materially. The weaker cultures changed little or not at all, but the more active ones lost much of their vigor; that is to say, long-continued growth under uniform conditions tended to reduce these cultures to a common level. It is not easy to restore this lost vigor. Repeated transfers in milk increased the activity of some of the cultures, but failed to bring the more active ones back to the rapid fermentation of the fresh cultures. It is probable that these differences are due to a variation in the vitality rather than to a variation in the particular function

of forming acid from sugar. In these studies it was frequently observed that those cultures curdling milk tardily or not at all multiplied slowly and never attained the numbers reached by the cultures curdling milk in a short time. This is illustrated by Table 2, in which is given the acid formation and rate of multiplication of two cultures, one curdling milk in a short time and one failing to curdle milk in 24 hours. Flasks of milk were inoculated from fresh milk cultures and incubated at 30° C.

TABLE 2.—*Relative rate of multiplication and acid formation in milk.*

Hours from inoculation.	Slow acid former.		Rapid acid former.	
	Acidity.	Bacteria per cubic centimeter.	Acidity.	Bacteria per cubic centimeter.
	Per cent.		*Per cent.*	
0	0.216	357,000	0.220	350,000
3	.216	586,000	.225	650,000
6	.225	1,690,000	.225	2,900,000
9	.225	16,900,000	.225	46,000,000
12	.230	59,500,000	.261	157,000,000
24	.306	710,000,000	.900	1,830,000,000

It will be observed that in a general way the acidity in each case is proportioned to the number of cells present. This is in accordance with the observation of Rahn,[10] who calculated the amount of acid formed in relation to the number of cells in the culture and found that this ratio was constant, although when only a small number of bacteria were present the amount of acid was so small that it could not be measured by ordinary methods. Schierbeck,[11] who studied this form of variation in the lactic-acid bacteria, found that by replating and making subcultures new cultures could be obtained, some of which followed the active fermentation of the original, while others were slow acid formers. In some of these cultures the rate of acid formation could not be varied by subsequent plating and selection, but cultures in which the ability to ferment lactose rapidly seemed to be fixed could be changed to slow fermenters by growing them in milk containing a small amount of carbolic acid. Buchanan and Truax [12] attempted to fix strains of the lactic-acid bacteria by selection and transfer from tubes of lactose broth showing wide differences in acidity. They failed entirely and concluded that "impressed variations do not appear to be heritable." It seems probable that they were unable to fix these variations because they were working, not with variations in a function, but with the rate of multiplication, something which may be controlled by an endless chain of circumstances. For the same reasons this characteristic can not be successfully used as a basis for classification.

GROWTH IN BROTH.

The growth in a medium of this nature should be considered as the expression of certain peculiarities not evident on superficial examination. A cloudiness or turbidity is, with the lactic-acid bacteria, an evidence of rapid growth, and it is governed more by external conditions than by the nature of the culture. Cultures which remain clear in nonsaccharine broth may be cloudy when certain sugars are added, and others may show cloudiness when dibasic potassium phosphate is added, but not in its absence. Profuse cloudiness is usually followed by a clearing and the accumulation of a sediment coinciding with the checking of the growth by increasing acidity. Some few cultures, however, never show turbidity, and the broth remains perfectly clear with no evidences of growth, although a high acidity is produced. This is perhaps due to the tendency to form tangled chains possessing in the aggregate a specific gravity greater than the broth. Müller [13] states that a clouded bouillon is associated with the formation of single cells or pairs, while a clear bouillon is coordinated with the formation of long chains. In his work he found that the cultures that never clouded the bouillon were the "Galt Stamme" found associated with yellow mammitis. When the failure to cloud the broth is fixed and constant it may be of some assistance in classification.

REDUCTION OF NITRATES.

The reduction of nitrates to nitrites was determined by growth in the following medium for 7 days at 30° C.

Peptone	gram..	1.0
Potassium nitrate	gram..	0.2
Water (distilled)	c. c..	1,000

Of the 147 cultures tested for the reduction of nitrates, 17 gave a positive reaction. All of these were differentiated from the typical lactic-acid bacteria by one or more coordinated characters. Twelve were of the liquefying tetrad group. Three (6fl, 7aa, and 7ak), were gas formers, and 7ak was in addition a bacillus larger than the lactic type and grew readily on solid media. One (7cc) resembled the lactic type in morphology but the cells were larger, and in addition to liquefying gelatin and giving an abundant growth on agar it made milk slimy. Culture 7dw was a small micrococcus.

None of the cultures belonging to Group I gave any evidence of growth in this medium. It is therefore of no value in making subdivisions of this group, but may be a convenient means for the detection of cultures resembling the type in many features but differing in certain salient points.

REDUCTION OF NEUTRAL RED.

In making this test the following medium was used:

Broth (neutral)	c. c..	1,000
Dextrose	grams..	5
One-half per cent solution Grübler's neutral red	c. c..	10

The neutral broth was made as follows:

Beef extract	grams..	4
Peptone	grams..	10
Water	c. c..	1,000

The tubes were examined after incubating at 30° C. for 7 days in an anaerobic jar from which the oxygen was exhausted by absorption with pyrogallic acid. Gordon[14] considers this test of diagnostic value. It has, however, the disadvantage of not always giving definite results, although with nearly all cultures the reduction was either nil or very evident. Of the 36 cultures reducing neutral red, a large proportion ferment the more resistant test substances, such as saccharose, glycerin, mannite, and raffinose, and 7, or 19 per cent of the whole, liquefy gelatin. None of those liquefying gelatin ferment raffinose, while of the 29 nonliquefying cultures reducing neutral red 75 per cent ferment raffinose. There is a correlation between the reduction of neutral red, the liquefaction of gelatin, and the fermentation of saccharose, glycerin, and mannite in one group and between neutral red, saccharose, glycerin, mannite, raffinose, and inulin in another. These correlations are evident in figure 6. The faculty of reducing neutral red seems to be usually coordinated with other reactions and is therefore of some value in differentiating cultures.

LIQUEFACTION OF GELATIN.

The value of this test is too generally recognized to need discussion. In our work we have used Clark and Gage's method of reducing the rate of liquefaction to mathematical terms, ignoring the appearance of the culture. The gelatin tubes were inoculated by spreading a few drops of a fluid culture on the surface of the medium. The line of the surface was marked on narrow strips of paper pasted on opposite sides of the tube, and the cultures were incubated at 18° to 20° C. At the end of 30 days the amount of liquefaction was measured and expressed as millimeters of liquefaction. The results of this test are tabulated in Table 3.

TABLE 3.—*The liquefaction of gelatin.*

Liquefaction.....millimeters..	0	1 to 5	6 to 10	11 to 15	16 to 20	21 to 25	Over 25
Number of cultures	108	3	10	10	2	3	2
Per cent of total	78.3	2.2	7.2	7.2	1.4	2.2	1.4

These results are platted in figure 3 to show the frequency of occurrence of certain arbitrary types.

This curve gives some indication of a division on the basis of gelatin liquefaction into three types, one failing to liquefy, one liquefying 6 to 15 millimeters, and one 20 to 25 millimeters. However, the total number of liquefying cultures was so small that it is safe to make a division into liquefiers and nonliquefiers only, and to depend on other tests for further division of the liquefiers. Even the separation of the liquefiers from the nonliquefiers is not entirely reliable, as it is well known that this character is variable and under some conditions may be entirely lost.

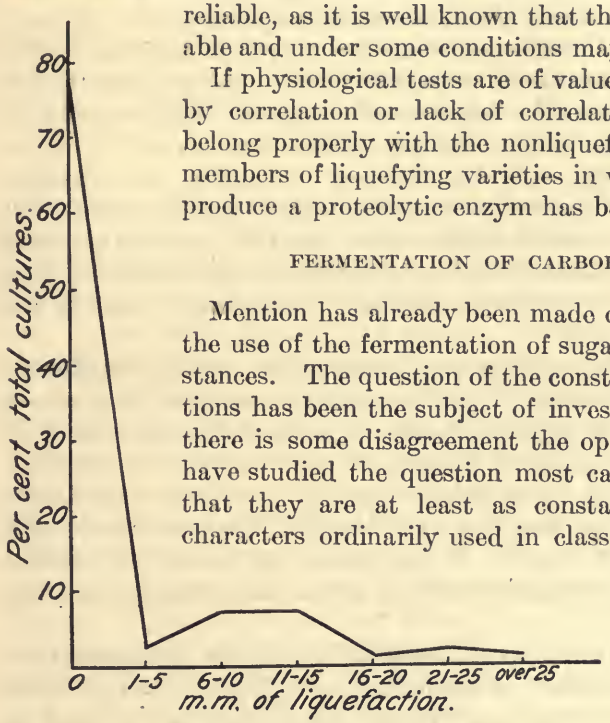

FIG. 3.—Frequency curve for gelatin liquefaction.

If physiological tests are of value they should show by correlation or lack of correlation which cultures belong properly with the nonliquefiers and which are members of liquefying varieties in which the ability to produce a proteolytic enzym has been lost.

FERMENTATION OF CARBOHYDRATES.

Mention has already been made of the objections to the use of the fermentation of sugars and similar substances. The question of the constancy of these reactions has been the subject of investigation, and while there is some disagreement the opinion of those who have studied the question most carefully seems to be that they are at least as constant as any of the characters ordinarily used in classification. Twort,[15] working with gas-forming cultures, was able to induce acid formation from sugars which the organism originally did not ferment by repeated transfers in a medium in which this sugar was the only carbohydrate furnished. Each transfer was held 14 days to allow the cells to work on the sugar after other sources of food had been exhausted. Ritchie[16] concluded that while cultures of *Bacillus coli* tested at different times gave constant fermentation reactions, the streptococci were inconstant. Gordon[17] tested the constancy of 11 cultures by passing them through mice. One culture lost ability to reduce neutral red and one gained ability to ferment salicin. All others remained unchanged. In all of this work the fermentative ability was determined by growing the organism in broth, with the addition of the test substance and litmus, and the change of the

litmus from blue was taken as a positive reaction. A slight change in the reaction of the medium may change litmus from blue to red, and this acidity may be formed from some substance other than the sugar. The reduction of the litmus is certainly not an indication of the fermentation of the test substance. The work of MacConkey [18] indicates that under natural conditions these reactions are constant and of value in differentiation. Among the cultures examined were 15 cultures of *B. typhosus* varying from one freshly isolated to one grown 16 years on artificial media. These gave identical fermentation reactions when tested with various carbohydrates.

In another paper the same investigator states that the fermentative reactions of *B. coli* remained unchanged after a long exposure to unfavorable conditions. He expresses the opinion that one group is not derived from another by the loss of characters.

Harding,[19] working with *Pseudomonas campestris*, an organism pathogenic to certain plants, obtained somewhat similar results. Of the four substances used for fermentation tests this organism attacked only one, but this and all other physiological tests employed were identical for the 44 cultures collected from various parts of the country.

In our own work no systematic investigation was undertaken to determine the constancy of the fermentation reactions, but all our observations tend to prove that the property of forming acid from carbohydrates and similar substances is not easily lost or acquired. One culture showing no evidence of ability to ferment saccharose was carried for 100 generations, or a period of about one year, on a saccharose-agar. At the end of this period the culture still showed no fermentation of saccharose and the lactose fermentation remained unchanged.

In no case did any of our cultures show any change in fermentative ability on repeated tests. It not infrequently happens that a culture failing entirely to give an acid reaction on the first test showed an active fermentation when the test was repeated, but this was evidently due to a failure of the inoculation rather than to a change in the organism. Many of the cultures grew so poorly on artificial media that they were propagated with difficulty and transfers frequently failed to grow. A large proportion of the cultures were subjected to these tests two or three times, some of them at intervals of several months. The second test almost always agreed with the first not only in the presence or absence of fermentation but also in the amount of acid formed. This is illustrated by Table 4, which contains results on the fermentation of lactose. These figures were picked at random.

TABLE 4.—*Showing constancy of fermentation of lactose.*

Test.	Acidity of broth expressed as per cent of lactic acid.										
First.....	0.112	0.171	0.216	0.387	0.288	0.000	0.180	0.306	0.153	0.108	0.252
Second.....	.117	.261	.171	.306	.225	.144	.261	.288	.135	.099	.243

With some of the test substances the reaction was always very positive; that is, the reaction remained unchanged or a considerable acidity was developed. This was especially noticeable with saccharose, mannite, and raffinose. With others, particularly with glycerin, the acid was developed slowly and in such small quantities that it was sometimes difficult to determine if there was a real fermentation or a slight change in the reaction which was independent of the test substance. In doubtful cases a retest usually gave definite results.

It is not to be expected that a character of this kind would be absolutely fixed. Indeed, the fact that one culture attacks a certain sugar while similar cultures do not is evidence that this function is or has been a variable one. There is, however, no evidence to show that the tendency toward variation in fermentation is any greater than in any other character used as a basis of classification. The greater difficulty comes in the interpretation of the results. The objection that the number of varieties obtained is limited only by the number of test substances used is valid only when an absolute separation is made on each individual reaction. The usual botanical scheme of dichotomous separation when applied to the classification of bacteria on the basis of fermentation tests leads only to confusion and the rejection of the system. In the card arranged for the classification of bacteria by a committee of the Society of American Bacteriologists, dextrose, saccharose, lactose, starch, and glycerin are used as test substances, and cultures are separated in the usual way on the fermentation of or failure to ferment any one substance. By the use of these test substances and similar methods we could separate our nonliquefying cultures into five varieties. But if it is proper to separate cultures on the basis of the fermentation of dextrose, saccharose, or lactose we can use also raffinose and galactose, and if glycerin is allowable mannite can not be excluded, while inulin may be as useful as starch. Adding these test substances and following the same principles of division, we obtain no less than 14 varieties, and even these are not stable, because the introduction of a new test substance would probably subdivide them still more minutely.

Gordon [10] was the first to make an extensive use of the fermentation tests. These tests were also used on an extensive scale by Andrewes and Gordon [20] and by Houston.[21] This work shows the possibilites of arranging a large number of cultures in groups around type sets of reactions. Not all of the cultures in each group agreed perfectly

with the type. Some failed in one reaction, while others possessed some character not common to the entire group. MacConkey,[18] using similar methods in a study of gas-forming bacteria from milk, was able to separate 112 cultures into 17 groups, and even these groups were sometimes separated by minor differences only.

On the basis of the individual reactions it was possible to separate these cultures into 64 varieties. This work was continued and placed on more scientific footing by Andrewes and Horder,[22] and especially by Winslow and Rogers, [23],[24] who applied the principles of biometry to the study of bacteria. In this way has been supplied a method of utilizing the physiological tests in such a way that bacteria may be collected in natural groups. In tabulating the characters of a large number of cultures, frequency of occurrence of those with certain common characters indicates the type, while the cultures varying from these types occur in smaller numbers and form the connecting links between the types. This represents the state of affairs in nature, while a description based by the ordinary method on the characters of a single culture may or may not agree with the type.

In our fermentation tests we have followed Winslow in determining the acidity rather than the mere fact of fermentation or nonfermentation. This is more exact and sometimes gives additional information of value in separating cultures. The medium was made as follows:

	Per cent.
Beef extract	0.4
Peptone	1.0
Dibasic potassium phosphate	.5
Test substance	2.0

The use of dibasic potassium phosphate is of advantage in that it serves to neutralize the acid and thus permits a more active growth and higher acid formation. The acid phosphate formed evidently checks the growth when a certain concentration is reached. The neutralization of culture media by this means is discussed by Henderson and Webster.[25]

The cultures were incubated 7 days at 30° C., with the exception of glycerin, which on account of the slow fermentation was held 14 days, and were titrated while cold against twentieth-normal sodium hydrate with phenolphthalein as an indicator. The result of the titration is expressed as per cent of lactic acid. Gas formation was determined by using an inverted inner tube in the dextrose broth. The results of the fermentation tests are given in detail in Table 1 and are recapitulated in Tables 5 and 6. The results shown in the two latter tables are given graphically in figures 4 and 5, in which the frequency of occurrence of cultures forming certain arbitrary amounts of acid is platted.

TABLE 5.—*Fermentation of test substances by liquefying cultures.*

Test substance.	Per cent of lactic acid.														
	Below 0.100.	0.101–0.150.	0.151–0.200.	0.201–0.250.	0.251–0.300.	0.301–0.350.	0.351–0.400.	0.401–0.450.	0.451–0.500.	0.501–0.550.	0.551–0.600.	0.601–0.650.	0.651–0.700.	Above 0.700.	Total cultures.
Dextrose:															
Number of cultures	1	4	5	12	1	0	3	1	0	1	0	0	1	0	29
Per cent of total...	3.45	13.8	17.2	41.4	3.45	0	10.3	3.45	0	3.45	0	0	3.45	0	.....
Lactose:															
Number of cultures	0	12	11	4	2	3	1	.....	.....	.....	.....	.....	.....	.....	33
Per cent of total...	0	36.4	33.3	12.1	6.0	9.1	3.0	.....	.....	.....	.....	.....	.....	.....	.....
Saccharose:															
Number of cultures	21	1	3	0	5	1	2	.....	.....	.....	.....	.....	.....	.....	33
Per cent of total...	63.6	3.0	9.1	0	15.1	3.0	6.1	.....	.....	.....	.....	.....	.....	.....	.....
Glycerin:															
Number of cultures	25	2	1	4	0	1	.....	.....	.....	.....	.....	.....	.....	.....	33
Per cent of total...	75.8	6.1	3.0	12.1	0	3.0	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mannite:															
Number of cultures	22	1	2	3	3	0	1	1	.....	.....	.....	.....	.....	.....	33
Per cent of total...	66.7	3.0	6.1	9.1	9.1	0	3.0	3.0	.....	.....	.....	.....	.....	.....	.....
Galactose:															
Number of cultures	17	4	5	3	2	0	1	.....	.....	.....	.....	.....	.....	.....	32
Per cent of total...	53.1	12.5	15.6	9.4	6.3	0	3.1	.....	.....	.....	.....	.....	.....	.....	.....
Raffinose:															
Number of cultures	31	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	31
Per cent of total...	100	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Inulin:															
Number of cultures	22	0	0	0	0	0	1	.....	.....	.....	.....	.....	.....	.....	23
Per cent of total...	95.6	0	0	0	0	0	4.4	.....	.....	.....	.....	.....	.....	.....	.....

TABLE 6.—*Fermentation of test substances by nonliquefying cultures.*

Test substance.	Per cent of lactic acid.														
	Below 0.100.	0.101–0.150.	0.151–0.200.	0.201–0.250.	0.251–0.300.	0.301–0.350.	0.351–0.400.	0.401–0.450.	0.451–0.500.	0.501–0.550.	0.551–0.600.	0.601–0.650.	0.651–0.700.	Above 0.700.	Total cultures.
Dextrose:															
Number of cultures	1	0	2	4	10	12	30	13	10	12	11	5	3	3	116
Per cent of total...	0.9	0	1.7	3.4	8.6	10.3	25.9	11.2	8.6	10.3	9.5	4.3	2.6	2.6	.....
Lactose:															
Number of cultures	3	5	5	8	16	17	30	31	1	0	0	1	.....	.....	117
Per cent of total...	2.6	4.3	4.3	6.8	13.7	14.5	25.6	26.5	0.8	0	0	0.8	.....	.....	.....
Saccharose:															
Number of cultures	75	0	0	1	2	1	3	8	7	15	3	1	1	.....	117
Per cent of total...	64.1	0	0	0.8	1.7	0.8	2.6	6.8	5.9	12.8	2.6	0.8	0.8	.....	.....
Glycerin:															
Number of cultures	93	2	12	7	4	1	.....	.....	.....	.....	.....	.....	.....	.....	119
Per cent of total...	78.2	1.7	16.8	5.9	3.4	0.8	.....	.....	.....	.....	.....	.....	.....	.....	.....
Mannite:															
Number of cultures	78	1	0	3	4	4	3	0	1	11	11	.....	.....	.....	116
Per cent of total...	67.2	0.9	0	2.6	3.5	3.5	2.6	0	0.9	9.5	9.5	.....	.....	.....	.....
Galactose:															
Number of cultures	8	5	8	19	28	20	16	11	1	1	1	.....	.....	.....	118
Per cent of total...	6.8	4.2	6.8	16.1	23.7	16.9	13.6	9.3	0.8	0.8	0.8	.....	.....	.....	.....
Raffinose:															
Number of cultures	90	0	1	1	0	0	0	1	1	0	1	12	7	1	114
Per cent of total...	78.9	0	0.9	0.9	0	0	0	0.9	0.9	0	0.9	10.5	6.1	0.9	.....
Inulin:															
Number of cultures	101	0	0	2	9	5	0	.....	.....	.....	.....	.....	.....	.....	117
Per cent of total...	86.3	0	0	1.7	7.7	4.3	.....	.....	.....	.....	.....	.....	.....	.....	.....

We have reckoned any acidity of below 0.1 per cent as no fermentation, although this may be an arbitrary distinction.

In the work by Winslow previously cited the frequency curves usually showed three modes. In his work, however, a larger number of cultures selected from various sources was used, while ours

came from milk only. Our curves usually show only two modes, one at the point of no fermentation and one at a point of acidity varying with the different test substances. There are, however, certain differences in the curves of the liquefiers and the nonliquefiers. The dextrose curve for the liquefiers shows that a large proportion form 0.2 to 0.25 per cent of acid, with a smaller number at 0.35 to

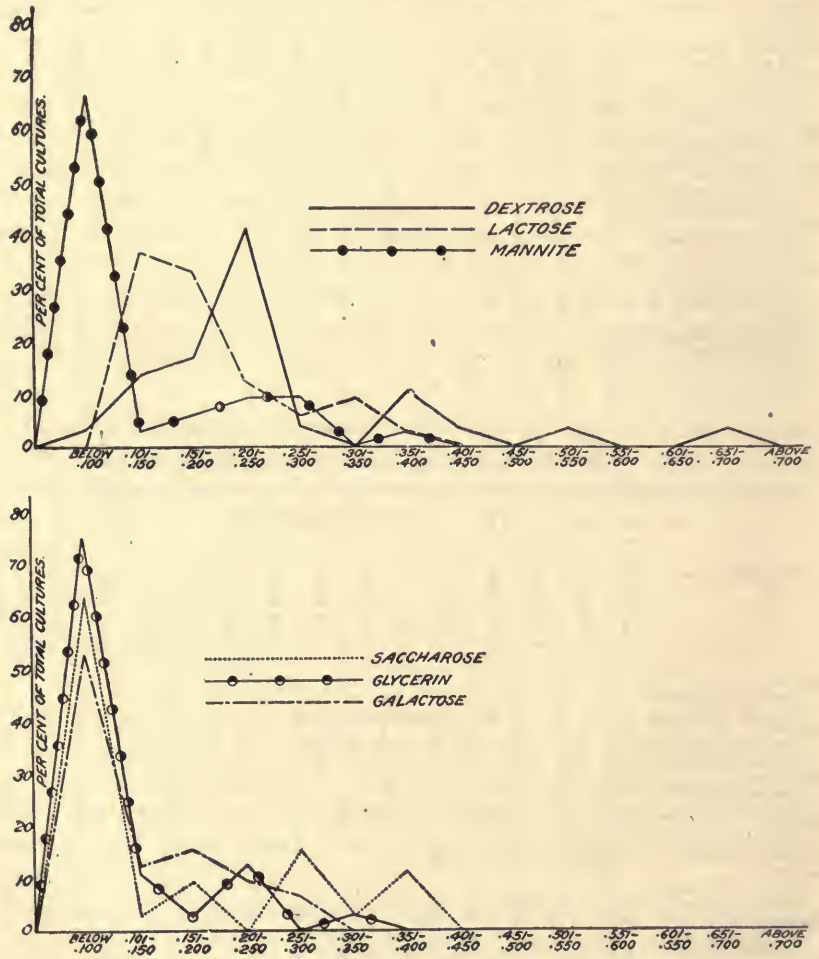

FIG. 4.—Frequency curves for acid formation by the liquefying cultures.

0.4, while with the nonliquefiers there is a high point at 0.35 to 0.4 per cent and possibly a second mode at 0.5 to 0.6.

With the liquefiers the other test substances show two modes only. Raffinose was not fermented at all, and only one culture formed acid from inulin.

The number of liquefying cultures was too small to make many deductions therefrom, but it is easy to separate these cultures into

two distinct groups. One of these forms a small amount (0.1 to 0.3 per cent) of acid from dextrose, and is a micrococcus usually appearing in tetrads. The effect on milk is weak, and the curdling, which is slow, is probably due more to the action of a rennet than to the production of acid. This group, as represented by these cultures, is undoubtedly heterogeneous, and by the application of these methods

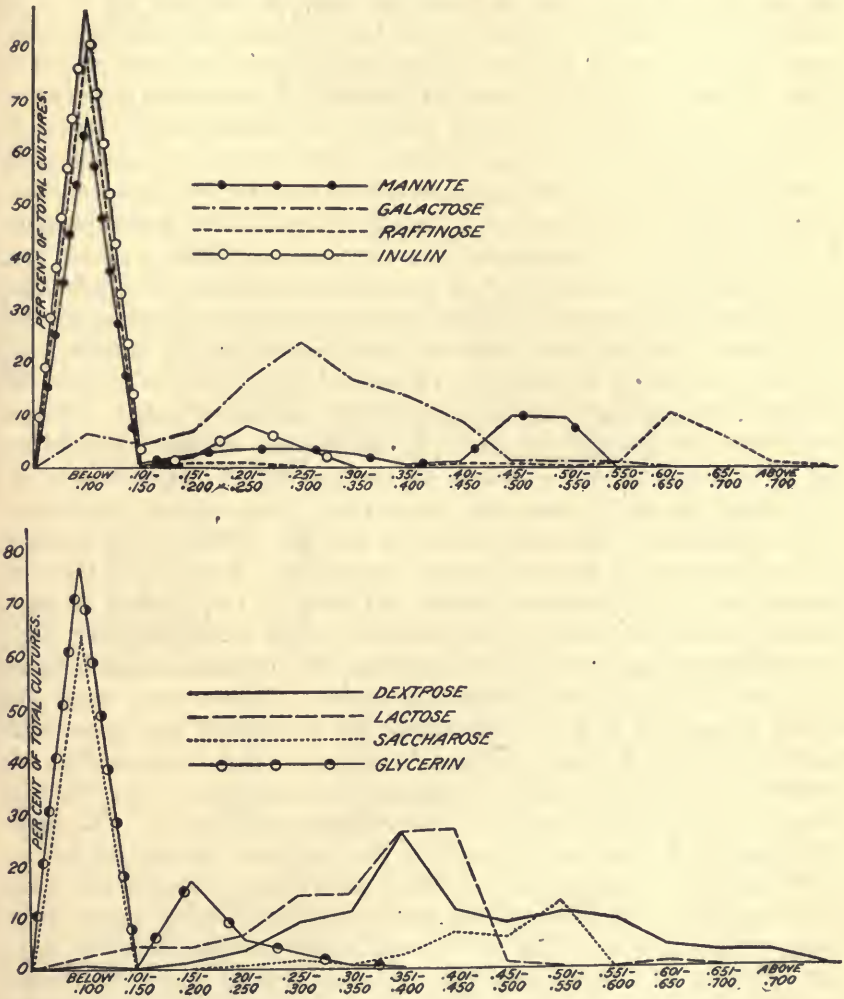

FIG. 5.—Frequency curves for acid formation by the nonliquefying cultures.

to a larger number of cultures would be split up in distinct subgroups. The second group of liquefiers is interesting in that it evidently is a variation from the typical nonliquefying lactic-acid organism. In its morphology it is identical with the ordinary type, but differs from it not only in the liquefaction of gelatin, but also in usually fermenting glycerin. Its action on milk is characteristic.

The milk is curdled promptly with a firm acid curd; digestion begins at once and almost always causes a separation of curd from the whey down the side of the tube.

In the nonliquefying group there are with all the test substances only two distinct modes in the curves with the possible exception of mannite. In this case there are three modes, one below 0.1 per cent, one between 0.1 and 0.35, and another between 0.45 and 0.5.

Reference to Table 1 shows that of the 8 cultures belonging to the group falling between 0.1 and 0.35 per cent, 2 were gas formers and 1 made milk slimy, while the other 5 apparently did not differ from those forming a high acidity or failing entirely to ferment this substance.

In arranging these reactions on the basis of their correlations, one of the formulas for the expression of correlation, as, for instance, that of Yule, may be used, but for this particular work the determination of the coefficient of correlation is not necessary. Even a casual examination of Table 1 shows that the nonliquefiers may be separated into two groups, in one of which the fermentation is usually limited to dextrose, lactose, and galactose, with an occasional culture fermenting saccharose or mannite. A second group may be formed of cultures in which the fermentative ability is distinctly higher. These groups are illustrated by figure 6, in which each culture is placed in one of four groups and arranged on the positive or negative side of a dividing line, as the case may be, in each of the salient characters.

The division of the nonliquefiers is not an arbitrary one, as the distinction between the two groups is marked. Not only is there a general lack of fermentative ability in Group A, but there is no correlation in the few cases of fermentation of the more difficultly fermentable substances. The fermentation of saccharose is no indication that the culture will ferment mannite. On the other hand, in Group B more of the test substances are fermented and there is a high correlation between certain activities. The fermentation of raffinose is usually correlated with the fermentation of saccharose, mannite, and glycerin, and to a lesser degree with inulin. The high fermentation is also correlated with the reduction of neutral red.

In making up these groups it was found that 6 cultures (6fl, 7aa, 7ak, 7cq, 7dw, and 13u) did not belong in this collection, and they were not included in the table. It will also be noticed that 7ci, 7cg, and 7dm are in a transition stage between Groups A and B, either through the loss of properties formerly possessed or the acquisition of new ones. In morphology and general culture characters all of the numbers of this group agree with the typical lactic culture. It should be stated that we obtained all of the cultures of Group B from one locality, Albert Lea, Minn., although not from one sample. However, in the course of another investigation a large number of cultures which could be properly placed in this group have been isolated from Washington milk, indicating that it is widely distrib-

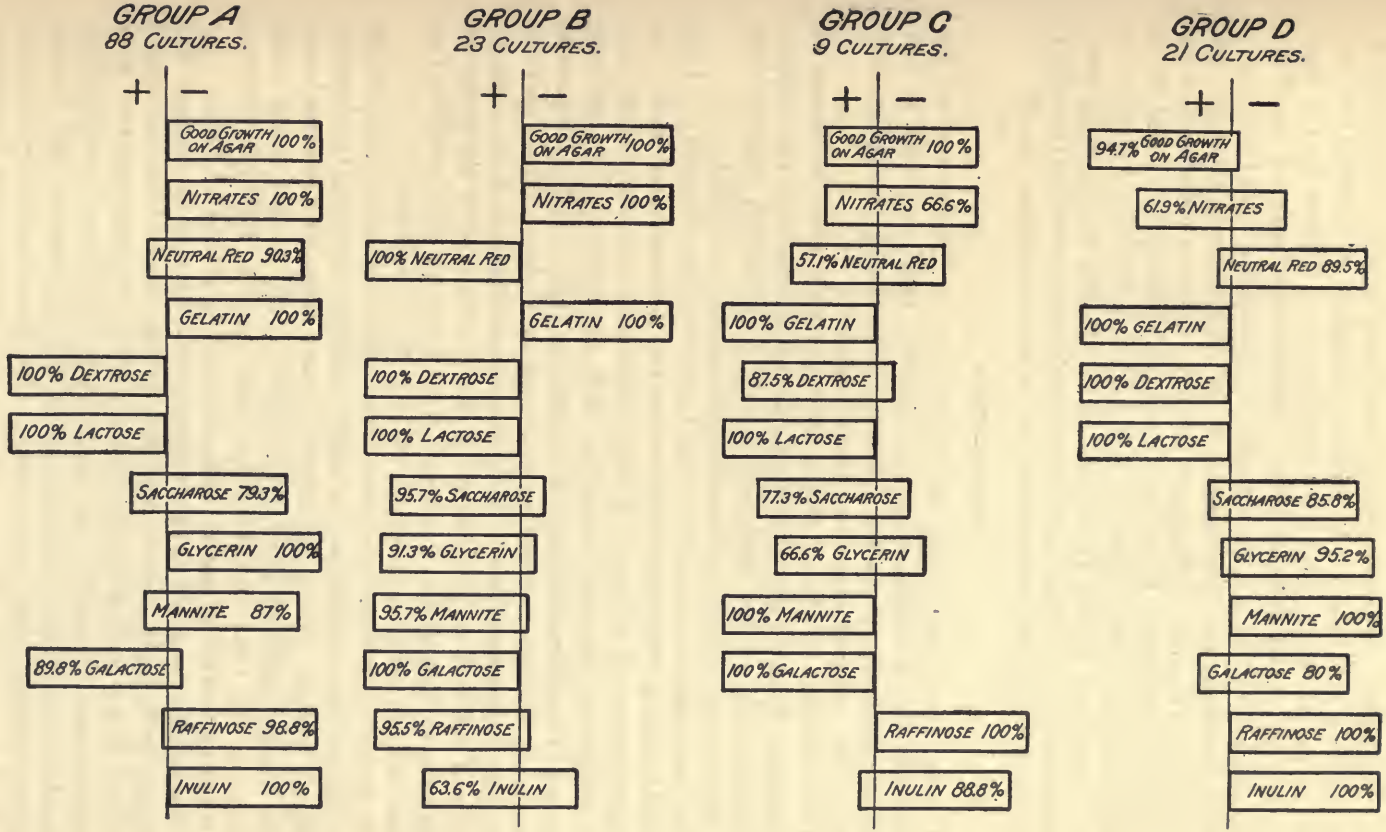

FIG. 6.—Grouping of cultures and distribution of characters in groups.

uted, although it does not occur in so large numbers as the Group A type. While the number of cultures included in Group C is too small to permit many positive deductions, it is evident that it represents a type quite distinct from A and B, from which it is differentiated not only by the liquefaction of gelatin but also by the correlated functions of the fermentation of mannite and glycerin and the failure to ferment raffinose and inulin. These 9 cultures include 3 differing somewhat from the others morphologically, and it is probable that a larger collection would allow a deeper and more positive separation. Group D is made up of cultures which, while they are of common occurrence in milk, have such a low fermentative ability that they probably take little part in the normal souring of milk.

CONCLUSIONS.

The stability of the fermentation tests is made evident not only by the constancy of the reactions on repeated tests, but also by the marked correlation between different fermentative activities and between the fermentations and other characters.

The usefulness of these tests is only apparent when by means of biometrical methods the correlations are established and the cultures are arranged in groups possessing certain characters in common, but in which minor variations from the type are not excluded.

The test substances used can not be determined arbitrarily. It is probable that it will be desirable to vary the test substances used with different groups of bacteria. We have found raffinose and glycerin and the gelatin test especially valuable, while saccharose, which has long been used for differential tests, has much less value. All of the groups have many cultures fermenting this sugar, and there is little correlation with other reactions. While the determination of the fermentation of raffinose or glycerin gives one a good idea of the group in which the culture should be placed, the knowledge that a culture ferments or fails to ferment saccharose is of little assistance.

It should be remembered that these cultures were all selected on the basis of the possession of a single positive character, the fermentation of lactose. If the collection had been made on a broader basis, it is highly probable that the cultures would have formed other groups around types distinct from those we have found but related to them by certain common characters and by transition forms.

The results recorded in this paper are too meager to warrant any attempt at fixing names or establishing the place of the lactic-acid bacteria in the bacteriological system, but we believe that this work indicates that future efforts in the direction of systematic bacteriology should be toward the determination of those characters that are significant and enduring rather than in fruitless controversy over the priority or stability of some name based on descriptions so undeterminative that they convey no meaning.

REFERENCES TO LITERATURE.

1. Hastings, E. G. A preliminary note on a group of lactic acid bacteria not previously described in America. Science, vol. 28, no. 723, p. 656. New York, Nov. 6, 1908.
2. Hastings, E. G., and Hammer, B. W. The occurrence and distribution of a lactic acid organism resembling the *Bacillus bulgaricus* of yogurt. Wisconsin Agricultural Experiment Station, Research Bulletin 6, pp. 197–206. Madison, June, 1909.
3. Heinemann, P. G., and Hefferan, Mary. A study of *Bacillus bulgaricus*. Journal of Infectious Diseases, vol. 6, no. 3, pp. 304–318. Chicago, June 12, 1909.
4. McDonnell, Milton Earle. Über Milchsäure–Bakterien. Inaugural Dissertation, Kiel, 1899.
5. Weigmann, H. Versuch einer Einteilung der Milchsäurebakterien der Molkereigewerbes. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 5, no. 24, pp. 825–831; no. 25, pp. 859–870. Jena, Dec. 5, 15, 1899.
6. Müller, Paul Th. Über die Streptokokken der Milch. Archiv für Hygiene, vol. 56, no. 1/2, pp. 90–107. Munich, 1906.
7. Löhnis, F. Versuch einer Gruppierung der Milchsäurebakterien. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 18, no. 4/6, pp. 97–149. Jena, Mar. 14, 1907.
8. Conn, H. W., Esten, W. M., and Stocking, W. A. A classification of dairy bacteria. Connecticut (Storrs) Agricultural Experiment Station, Eighteenth Annual Report (1906), pp. 91–203. Middletown, 1907.
9. Heinemann, Paul Gustav. The kinds of bacteria concerned in the souring of milk. Inaugural Dissertation, University of Chicago. Chicago, 1907.
10. Rahn, Otto. The fermenting capacity of the average individual cell (*Bacterium lactis acidi*). Science, vol. 33, no. 849, pp. 539–540. New York, Apr. 7, 1911.
11. Schierbeck, N. P. Ueber die Variabilität der Milchsäurebakterien mit Bezug auf die Gärungsfähigkeit. Archiv für Hygiene, vol. 38, no. 3, pp. 294–315. Munich, 1900.
12. Buchanan, Robert Earle, and Truax, Roy. Noninheritance of impressed variations in *Streptococcus lacticus*. Journal of Infectious Diseases, vol. 7, no. 5, pp. 680–697. Chicago, Oct. 25, 1910.
13. Müller, Leo. Vergleichende Untersuchungen über Milchsäurebakterien (des Typus Güntheri) verschiedener Herkunft, nebst Beitrag zur Fräge der Stellung dieser Organismen zu den typischen Streptokokken. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, Abteilung 2, vol. 17, no. 14/16, pp. 468–479. Jena, Dec. 7, 1906.
14. Gordon, M. H. Report of some characters by which various streptococci and staphylococci may be differentiated and identified. Great Britain. Local Government Board, Thirty-third Annual Report (1903–04), supplement containing Report of the Medical Officer (1903–04), pp. 388–430. London, 1905.
15. Twort, F. W. The fermentation of glucosides by bacteria of the typhoid-coli group and the acquisition of new fermenting powers by *Bacillus dysenteriæ* and other micro-organisms. Preliminary communication. Proceedings of the Royal Society of London, series B, vol. 79, no. B532, pp. 329–336. London, June 5, 1907.

16. Ritchie, John. Notes on experiments as to the constancy of the carboyhdrate reactions of the streptococci. Lancet, vol. 175, no. 4432, pp. 374–376. London, Aug. 8, 1908.
17. Gordon, M. H. A ready method of differentiating streptococci, and some results already obtained by its application. Lancet, vol. 169, no. 4289, pp. 1400–1403. London, Nov. 11, 1905.
18. MacConkey, Alfred. A contribution to the bacteriology of milk. Journal of Hygiene, vol. 6, no. 3, pp. 385–407. Cambridge, July, 1906.
19. Harding, H. A. The constancy of certain physiological characters in the classification of bacteria. New York Agricultural Experiment Station, Technical Bulletin 13. Geneva, June, 1910.
20. Andrewes, F. W., and Gordon, M. H. Report on the biological characters of the staphylococci pathogenic for man. Great Britain, Local Government Board, Thirty-fifth Annual Report (1905–06), supplement containing the report of the Medical Officer (1905–06), pp. 543–560. London, 1907.
21. Houston, A. C. Report on the bacteriological examination of the normal stools of healthy persons. Great Britain, Local Government Board, Thirty-third Annual Report (1903–04), supplement containing Report of the Medical Officer (1903–04), pp. 472–527. London, 1905.
22. Andrewes, F. W., and Horder, T. J. A study of the streptococci pathogenic for man. Lancet, vol. 171, no. 4333, pp. 708–713; no. 4334, pp. 775–782; no. 4335, pp. 852–855. London, Sept. 15, 22, 29, 1906.
23. Winslow, C. E. A., and Rogers, Anne F. A revision of the Coccaceæ. Science, vol. 21, no. 539, pp. 669–672. New York, Apr. 28, 1905.
24. Winslow, Charles Edward Amory, and Winslow, Anne Rogers. The systematic relationships of the Coccaceæ. New York, 1908.
25. Henderson, Lawrence J., and Webster, H. B. The preservation of neutrality in culture media with the aid of phosphates. Journal of Medical Research, vol. 16, no. 1, pp. 1–5. Boston, Mar., 1907.

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